ERK signaling and amphetamine-induced potentiation of conditioned cue effects on reward seeking

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Reward-seeking behaviors can be potentiated by exposure to cues that have been paired with a reward. This phenomenon is called Pavlovian instrumental transfer (PIT). PIT-like effects are thought to contribute to relapse of drug intake upon exposure to drug-associated cues. PIT was shown to be enhanced by prior exposure to psychostimulants; however, the molecular mechanisms involved are unknown. We previously found that extracellular signal-regulated kinase (ERK) activation within the nucleus accumbens (NAc) critical for PIT. Here we examine a possible involvement of NAc ERK signaling in the potentiation of PIT by prior exposure to psychostimulants. Rats underwent Pavlovian conditioning to associate a tone with food delivery, then underwent instrumental training to press a lever for food, and finally were tested for PIT. After each Pavlovian session half of the rats were treated with amphetamine (1mg/kg; i.p.) and the rest with saline (1ml/kg; i.p.). Some rats received Pavlovian conditioning only to assess the effect of prior amphetamine exposure specifically on cue-evoked ERK activation in the NAc. To determine the importance of timing of amphetamine exposure in relation to the Pavlovian conditioning training, some rats were treated with drug or saline 6 hrs after the daily session. Amphetamine treatment after daily Pavlovian training increased cue-evoked NAc ERK activation without affecting basal ERK activation or discriminative food cup approach. Amphetamine exposure caused a

marked increase in PIT accompanied by an increase in cue-evoked ERK activation. There were no drug effects on basal lever pressing, inactive lever pressing, or discriminative food cup approach during the PIT test. The effect of prior amphetamine exposure on cue-evoked NAc ERK activation and PIT were observed when amphetamine was administered immediately after the daily Pavlovian conditioning but not when it was administered 6 hrs later. These findings are consistent with a role for cue-evoked NAc ERK activation in the enhancement of PIT observed days after repeated exposure to amphetamine. Potentiation dependence on the timing of amphetamine administration relative to Pavlovian conditioning argues against an explanation in terms of general sensitization but instead suggests a drug effect on the consolidation of the cue-reward association.

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List of abbreviations

AMPH	amphetamine
BLA	basolateral amygdala
CS	conditioned stimulus
D1R	dopamine receptor type 1
D2R	dopamine receptor type 2
DA	dopamine
ERK	extracellular signal-regulated kinase
FSCV	fast scan cyclic voltammetry
kg	kilogram
MEK	mitogen-activated protein kinase kinase
mg	miligram
NAc	nucleus accumbens
PFC	prefrontal cortex
PIT	Pavlovian instrumental transfer
SAL	saline
VTA	ventral tegmental area
NMDA-R	N-methyl-D-aspartate receptor

1.0 INTRODUCTION

Drug addiction is defined as a chronic disorder characterized by compulsive drug seeking that persists despite harmful consequences (Leeman & Potenza, 2012). Relapse is a major problem in the treatment of drug abuse, and can be triggered by either stress, re-exposure to the drug, or, most commonly, exposure to drug-associated cues (Volkow & Kalivas, 2005; Leeman & Potenza, 2012; Koob & Volkow, 2010; McLellan, 2000). The economic cost to the U.S. society due to substance abuse and addiction has been estimated to be about $559 billion/year (health care, productivity loss, crime, incarceration, and drug enforcement) (Office of National Drug Control Policy, 2004). Understanding how cues affect behavior may have a large impact on the success of treatment of drug abuse.

Environmental cues play an important role in learning adaptive behaviors; through associative learning of the relation between cues and outcomes, organisms gain knowledge that enables them to increase survival (e.g., seeking out positive outcomes while avoiding negative outcomes) (Cardinal et al., 2002). Pavlovian conditioning is the mechanism by which a previously neutral stimulus that has repeatedly been paired with a specific outcome, such as a reward, comes to predict the outcome and elicits a response, i.e., it becomes a conditioned stimulus (CS). CSs that

predict reward can potentiate behaviors that are aimed at obtaining the reward (i.e., reward seeking). The excitatory effect exerted by CSs on reward seeking is called Pavlovian-Instrumental Transfer (PIT) (Day & Carelli, 2007; Dickinson & Balleine, 1994; Holmes et al., 2010; Lovibond, 1983). Association between environmental cues and drugs is thought to be a critical factor in drug addiction: PIT-like effects have been hypothesized to contribute to drug abuse because exposure to drug-associated stimuli (e.g., a drug syringe) can increase the likelihood of relapse (Everitt et al., 2001; Volkow & Kalivas, 2005; Everitt et al., 2008; Childress et al., 1993; Leyton, 2007; O'Brien et al., 1998; Semenova & Markou, 2003; Bassareo et al., 2011).

Cue-reward associative learning and PIT both have been shown to depend on brain regions that form the mesolimbic reward system, including the ventral tegmental area (VTA), the nucleus accumbens (NAc), the prefrontal cortex (PFC), and the basolateral amygdala (BLA) (Cardinal et al., 2002; Chang et al., 2012; Homayoun & Moghaddam 2009; Corbit et al., 2007; Sesack & Grace, 2010; Roitman et al., 2005). Within the mesolimbic reward system, the NAc was shown to be of particular importance for PIT. Studies using either excitotoxic lesions or temporary inactivation by muscimol have shown that the core subregion of the NAc is critical primarily for general PIT, whereas the shell subregion of the NAc is important for outcome-specific PIT (Shiflett & Balleine, 2010; Corbit & Balleine, 2011). General PIT refers to the general motivating effect a CS can have on reward seeking. General PIT is typically established in the laboratory by using only one CS, one reward, and one action to obtain the reward. Outcome-selective PIT refers to the effect observed when a CS potentiates only the action that is aimed to obtain the same reward as the one predicted by the CS (Cardinal

et al., 2002). We study PIT using a protocol that mixes aspects from both general and outcome-specific PIT, and we have observed previously a similar pattern of activation in the two subregions of the NAc during PIT (Remus & Thiels, 2013).

PIT has been shown to depend on dopamine (DA) receptor activation within the NAc. Systemic treatment with flupenthixol, a general non-selective dopamine receptor antagonist, blocked PIT (Dickinson et al., 2000; Wassum et al., 2011), and experiments with specific antagonists of either D1 receptors (D1R, SCH23390) or D2 receptors (D2R, Raclopride) infused into the NAc also demonstrated blockade of PIT. The latter findings strongly suggest that DA signaling through these receptors is required for PIT (Lex & Hauber, 2008). Experiments using fast scan cyclic voltammetry (FSCV) revealed an increase in phasic DA release within the NAc during PIT, where the increase in DA was time-locked to the cue presentation and also correlated with lever-press rate (Wassum et al., 2013). Likewise, exposure to drug-associated cues causes a rise in extracellular DA levels in the NAc (Owesson-White et al., 2009). Seemingly, a rise in DA levels during cue presentation is critical for PIT.

Accumulating evidence indicates that psychostimulants have the ability to enhance PIT (Wyvell & Berridge, 2000; Wyvell & Berridge, 2001; Saddoris et al., 2011; Shiflett, 2011; Peciña et al., 2013; LeBlanc et al., 2013). Self-administration of cocaine was found to potentiate PIT. Briefly, rats were trained in Pavlovian and instrumental conditioning and then were allowed to self-administer cocaine for 14 days. PIT was tested one week after the end of the self-administration period (Saddoris et al., 2011) Similarly, experimenter-delivered cocaine (15 mg/kg for six days; LeBlanc et al., 2013) or amphetamine (3 mg/kg for six days; Wyvell & Berridge, 2000; Wyvell & Berridge,

2001) were found to potentiate PIT. So far, a variety of protocols of chronic psychostimulant exposure can potentiate PIT.

We have showed that PIT depends critically on the activation in the NAc of a critical molecule involved in synaptic plasticity, learning, and memory -extracellular signal-regulated kinase (ERK) (Shiflett et al., 2008). Blockade of ERK activation by a MEK/ERK inhibitor prevents PIT, without affecting baseline lever pressing or conditioned approach during the PIT test. Our study also showed that ERK activation is specifically triggered by exposure to a reward-associated cue. Increasing DA signaling by intra-ventricular infusion of the D1R agonist SKF82958 was found to increase extracellular regulated-signal kinase (ERK) activation in the NAc. (Haberny & Carr, 2005). And furthermore, D1R antagonist injection can prevent the ERK activation induced by amphetamine (Shi & McGinty, 2011). Increase in cue-evoked ERK activation can also be linked to reports of cue-evoked DA transients in NAc (Wassum et al., 2013) and it has been shown that repeated exposure to psychostimulants can alter NAc DA regulation (Nishikawa et al., 1983). The increase in PIT after repeated psychostimulant exposure may be the result of increased cue-evoked DA transients. In light of the positive coupling between D1R activation and ERK activation (Haberny & Carr, 2005), and the critical role of ERK in PIT (Shiflett et al., 2008), the enhancement of PIT after chronic psychostimulant may be mediated through increased cue-evoked ERK activation, probably through altered DA regulation.

Psychostimulants have been shown to enhance working memory (Spencer et al., 2012) and associative memories (Wood & Anagnostaras, 2009; Simon & Setlow, 2006). Low doses of amphetamine were shown to enhance memory of the CS during

Pavlovian fear conditioning (Wood & Anagnostaras, 2009). Furthermore, intermittent amphetamine exposure immediately after Pavlovian training enhanced memory consolidation of the cue-reward association. This effect was observed only with immediate exposure to amphetamine, but not when the drug was administered two hours after Pavlovian training (Simon & Setlow, 2006). Additionally, psychostimulants can cause transient increases in activation throughout the brain ERK (Mao et al., 2012; Valjent et al., 2000; Valjent et al., 2004). This transient increase may strengthen the consolidation of experiences that immediately preceded the exposure to psychostimulants. Associative memory formation, as occurs in fear conditioning or spatial learning, is accompanied by an increase in ERK activation in brain regions critical for consolidation of the associative memory (Sweatt, 2001; Adams & Sweatt, 2002; Ying et al., 2002; Kelly et al., 2003). Moreover, consolidation of associative memory, as well as forms of synaptic plasticity hypothesized to underlie memory consolidation, was shown to depend on ERK activation (Satoh et al., 2007; Cerovic et al., 2013; Kelly et al., 2003). Systemic or brain region-specific pharmacological inhibition of ERK was found to interfere with both long-term potentiation and long-term depression of synaptic strength (Cerovic et al., 2013; English & Sweatt, 1997; Valjent et al., 2006), and to cause deficits in consolidation of tone-shock and object or spatial information (Schafe et al., 2000; Kelly et al., 2003). The requirement of ERK activation for the consolidation of object recognition memory is transient, because inhibition of ERK hours after the learning experience was shown not to interfere with the retention and retrieval of the memories (Kelly et al., 2003). Thus, ERK activation appears to be critically involved only in early memory consolidation. If inhibition of ERK interferes with

associative memory consolidation, then boosting (i.e., increasing) ERK action may strengthen memory consolidation (Mazzucchelli et al., 2002). In this sense, exposure to psychostimulants immediately after a Pavlovian conditioning experience may lead to a stronger (more robust) CS-outcome association than exposure to psychostimulants hours after the associative experience.

The goals of the present experiments were to examine (1) the relation between cue-evoked ERK activation in the NAc and potentiation of reward-seeking behavior observed after pre-exposure to psychostimulants, and (2) the role of the timing of amphetamine exposure in relation to Pavlovian conditioning in the psychostimulant effect on cue-evoked ERK activation and PIT. To achieve these goals, I addressed the following specific aims:

Aim 1: To determine whether amphetamine exposure after daily Pavlovian training increases NAc ERK2 activation evoked by a Pavlovian conditioned cue in the drug-free state. Rats were trained to associate a tone with food delivery and were injected with either amphetamine (AMPH, 1mg/kg) or saline (SAL, 1ml/kg) immediately after each daily Pavlovian training session. Testing of Pavlovian conditioned approach was performed in extinction 48 hrs after the last drug injection, and CS-evoked ERK2 activation in the NAc was assessed by Western blot analysis.

Aim 2: To determine whether potentiation of the PIT effect after psychostimulant pre-exposure is accompanied by an increase in CS-evoked NAc ERK2 activation. Rats were injected with either AMPH or SAL immediately after each daily Pavlovian training session as described above, and then received instrumental training under drug-free

conditions. PIT testing was performed in extinction, also under drug-free conditions. Western blot analysis was used to assess NAc CS-evoked ERK2 activation.

Aim 3: To determine whether the enhancement of the increase in CS-evoked NAc ERK2 activation after amphetamine pre-exposure is sensitive to the timing of amphetamine treatment after Pavlovian training. Rats were injected with either AMPH or SAL after each daily Pavlovian training session; however in this case, administration took place 6 hrs after the end of the training session. Testing of Pavlovian conditioned approach was performed in extinction under drug-free conditions, and NAc CS-evoked ERK2 activation was assessed by Western blot analysis.

Aim 4: To determine whether potentiation of PIT and the associated enhancement of CS-evoked ERK activation in the NAc after psychostimulant pre-exposure is sensitive to the timing of amphetamine treatment after Pavlovian training. Rats were injected with either AMPH or SAL 6 hrs after each daily Pavlovian training session as described above, received instrumental training under drug-free conditions, and then were tested for PIT in extinction. NAc CS-evoked ERK2 activation was assessed by Western blot analysis.

2.0 METHODS

2.1 SUBJECTS AND BEHAVIORAL INSTRUMENTS

A total of 101 Sprague Dawley rats (Hilltop lab animals, Scottdale, PA) were used in this study. Rats weighing 250-275 g upon arrival were housed individually in isolating cages and supplied with ad libitum food and tap water. During the course of the experiment, rats were handled and weighed daily and were placed on a restricted diet of 14-16g of rat chow per day to maintain their body weight at approximately 90% of the free-fed weight of similar-aged rats. Training procedures took place in 6 instrumental chambers (30 cm x 23 cm x 23 cm; med associates, St. Albans, VT). Each chamber has a single house light, a floor with metal bars, and a speaker that delivers a 3-khz 80-db tone when activated. A food cup is mounted on the front wall and is attached to a pellet dispenser that releases a single 45-mg sucrose pellet (Bio-Serv, Frenchtown NJ) when activated. An infrared beam source and detector are mounted on either side of the food cup, and are used to record food cup-approach behavior. Each chamber also has 2 levers mounted on either side of the food cup. The chambers are housed in sound-attenuating boxes equipped with a background noise-generating fan (Bsr, Laurel, MD). Equipment is controlled through a desktop computer running med associates proprietary software (Med PC).

One protocol was designed to evaluate the effects of amphetamine on cue-induced ERK activation. A second protocol was designed to test whether amphetamine-induced potentiation of PIT is accompanied by an enhancement of cue-evoked ERK2 activation. In this protocol, rats underwent Pavlovian conditioning training followed by instrumental conditioning, and then received a PIT test. Rats were divided into four experimental groups: TF-Amph (tone-food paired, amphetamine treated), TF-Sal (tone-food paired, saline treated), TO-Amph (tone only, amphetamine treated) and TO-Sal (tone only, saline treated).

The behavioral data were collected in the rodent behavior analysis core of the University of Pittsburgh, Schools of Health Sciences. All animal procedures have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.1.1 Pavlovian conditioning

Rats were transported from their home cages to the testing room. Before training, rats were habituated to the conditioning chamber in a single 30-min session with only the lights on. Each pavlovian conditioning session began with illumination of the house light. During each session, rats received six presentations of a 3-khz 80 db tone that served as CS. Each tone was 90 sec in duration, and during the tone 3 sucrose pellets were delivered on a random time 20-sec schedule. Half of the trials (three) started delivering food 30 sec after the tone onset and were used for quantification of food cup approach; during the other 3 trials food delivery started at tone onset. The inter-stimulus interval varied between 3-5 min (isi; mean = 4.5 min). The training session ended by the termination of the house light. Rats performed one training session per day for 8

consecutive days (fig 2a). After each Pavlovian conditioning session, rats were treated with either Amph (1 mg/kg, i.p.) or Sal (1 ml/kg, i.p.) either immediately (0 hr; exp 1 and 2), or 6 hr (exp 3 and 4) after the end of the training session. Control rats for determination of basal NAc ERK2 activation received only tone presentations and no food during training (fig 2b). Control rats received either Amph or Sal treatment after the daily training sessions, similar to experimental rats. Forty-eight hours after the last session and drug treatment, all rats received four tone presentations in the absence of food (test). The number of food cup approaches (measured by infrared beam breaks) was counted for the 30 sec before tone onset (preCS) and during the first 30 sec of the tone (CS). Discrimination rate was calculated as food cup approaches during the CS minus the food cup approaches during the pre CS.

2.1.2 Instrumental conditioning

Training sessions began with illumination of the house light and insertion of both levers into the conditioning chamber. One lever (active lever), when pressed, delivered a single sucrose pellet into the food cup. Pressing the other lever (inactive lever) had no programmed consequences. The active lever was randomly assigned to the left or the right side and counterbalanced across rats. On the first day, rats received two sessions in which on average each active lever press resulted in delivery of a single pellet (fixed ratio 1; FR1). The following two days they received two sessions in which every three active lever presses (on average) resulted in delivery of one pellet (random ratio 3; RR3). Sessions were terminated after 50 pellets had been delivered. On the third day of instrumental conditioning, rats received two sessions of RR5. Each session was

terminated after 100 pellets had been delivered. During the last day of instrumental conditioning, rats received one RR5 session and one RR8 session (fig 2c). The pit test took place the next day and began with illumination of the house light and insertion of both levers into the instrumental chamber. The test was conducted in extinction (no pellets were delivered). After a 4.5 min interval, which served to partially extinguish the instrumental response, four 90-sec tones were presented, with a 3 min inter-trial-interval (iti) separating each tone presentation. Total session duration was 24 min. Number of active and inactive lever presses were recorded during the tone (CS) and during a 90-sec period preceding tone onset (preCS); the number of infrared beam breaks were also recorded during the first 30-sec of each tone presentation (CS) and during a 30-sec interval preceding tone onset (preCS). Pit effect was measured as higher rate of lever pressing during the CS compared to lever presses during the preCS.

2.2 WESTERN BLOT

Rats were anesthetized immediately following the test with an i.p. Injection of chloral hydrate (300 mg/kg dissolved in 0.9% NaCl) and decapitated. Brains were quickly flash-frozen in isopentane chilled on dry ice and stored at -80°c until further processing. Nucleus accumbens (NAc) samples were obtained from 1-mm thick slices collected between ~2.2 and ~1.2 mm anterior to bregma. The NAc samples were excised by placing a tissue punch (2 mm diameter; fine science tools) over and medial to the anterior commissure. Samples were homogenized in a buffer containing 150 mm NaCl, 1mm Edta, 50mm Tris ph 7.4, 0.05% sds, 1% triton-x, 1mm dithiothreitol (dtt), 1x

protease inhibitor cocktail set v, edta-free, 2 mm sodium fluoride, 1 mm orthovanadate, 2 mm sodium pyrophosphate, and 1 mg/ml pepstatin. The homogenate was centrifuged for 15 min at 14,000 rpm, the supernatant was collected, and samples were diluted with homogenization buffer to a uniform protein concentration. Homogenates were mixed with sample buffer (2.5 m tris ph=6.8, 40% glycerol, 8% sds, and 30 µg/ml dtt) and heated to 95˚C for 5 min. From each sample, 45 µg of protein were resolved electrophoretically on a 10% acrylamide/bisacrylamide gel through sds-page and then transferred to a PVDF immobilon membrane. All washes and solutions were made in tris-buffered saline (tbst: 0.05m tris ph 7.9, 0.15m NaCl, 0.1% tween-20). The membranes were washed and blocked for 1 hr at room temperature (RT) in 5% non-fat milk in tbst, and then incubated overnight at 4°C in a 5% bovine-serum albumin (BSA) solution containing an antibody that selectively recognizes both residues of phosphorylated ERK (1:2500 dilution; Cell Signaling, Beverly, MA). The membrane was washed again and then incubated in a HRP-linked secondary antibody (anti-rabbit, 1:5000; Cell Signaling, Beverly, MA). Bands were visualized with an enhanced chemiluminescence reagent (Lumiglo, Cell Signaling, Beverly, MA). Blot images were captured with a CCD camera (Hammatsu photonics, Japan) and analyzed using densitometry software (UVP Labworks, Upland, CA). The membranes then were stripped of their antibodies by incubation at 50˚C for 45 min in a solution containing 62.5 mm tris (ph 6.7), 2% SDS, and 0.62% β-mercaptoethanol. Membranes were re-probed with an antibody that recognizes both phosphorylated and unphosphorylated ERK and the same procedure as above was followed. The level of dual-phosphorylated (t183 and

y185) activated ERK2 (pERK2) was expressed as a ratio against levels of total ERK2 (tERK2; phosphorylated and unphosphorylated).

2.3 STATISTICAL ANALYSIS

Pavlovian and instrumental conditioning were analyzed by three-way analysis of variance (ANOVA), with day as within-subject factor, and drug (Sal vs Amph) and group (TF vs TO) as between-subject factors. Pavlovian test day and ERK2 activation were analyzed by two-way ANOVA with group and drug as between-subject factors. PIT test behavior was analyzed by three-way ANOVA, with CS period (CS vs preCS) as within-subject factor, and drug and group as between-subject factors. Post-hoc pairwise comparisons with the Bonferroni correction were used for analysis of specific differences in any cases where interactions were significant.

3.0 RESULTS

3.1 AMPHETAMNE PRE-EXPOSURE INCREASES CUE-EVOKED ERK2 ACTIVATION

To determine whether psychostimulant pre-exposure has an effect on CS-evoked NAc ERK2 activation, rats were treated with either amphetamine (1mg/kg i.p.) or saline (1 ml/kg i.p.) immediately after the daily Pavlovian training session. As shown in Figure 3A, at the first day of training, none of the four groups (TF-Amph, n=7; TF-Sal, n=7; TO-Amph, n=7 and TO-Sal, n=7) showed discriminative approach to the food cup (more food cup approaches during the CS period compared to the preCS). TF groups developed discriminative food cup approach during the eight days of training, but this effect did not differ between drug treatments, demonstrated by a significant group x day interaction ($F_{7,168}$ = 14.938; P<0.001) but no significant group x day x drug interaction ($F_{7,168}$ = 0.419; P=0.889). Post-hoc comparisons showed no difference in discriminative scores between groups on the first day of training. On the 8th and last day of training, means for TF-Amph and TF-Sal groups were significantly higher than those on day 1 (P<0.001), whereas the means of the TO-Amph and TO-Sal controls did not differ from the values on day 1. Two days after the last training and drug treatment, rats were tested in extinction. As expected, the level of discriminative food cup approach was

significantly higher among TF groups than TO groups (Figure 3B; main group effect, $F_{1,24}$ = 32.574; P<0.001), but it was not sensitive to prior drug treatment, as indicated by a lack of significant group x drug interaction ($F_{1,24}$ = 0.284; P=0.599). Trial-by-trial analysis of discriminative food cup approach during Pavlovian testing (Figure 3C), however, revealed a slight difference between the groups. Whereas discriminative approach to the food cup did not differ between the TF-Sal and the TF-Amph groups on the first trial of the four-trial test (Student's t-test for independent groups, t_{12} =-0.86, p=0.404, two-tailed), it was significantly lower by the TF-Sal group than the TF-Amph group on the fourth trial (t_{12} =-3.5, p=0.004, two-tailed).

Immediately after the test, brains were collected to asses ERK2 activation (i.e., pERK2 immunoreactivity relative to tERK2 immunoreactivity in the same sample). Figure 4 shows that ERK2 activation in NAc tissue of the TF groups was higher than ERK2 activation in tissue samples of the TO groups. Importantly, the level of ERK2 activation was higher for rats in the TF-Amph group than rats in the TF-Sal group. ANOVA showed a main effect of group ($F_{1,24}$ = 69.174, P<0.001) and a significant group x drug interaction ($F_{1,24}$ = 4.462, P=0.045). Post-hoc comparisons confirmed that ERK2 activation was significantly higher in the TF-Sal group than the TO-Sal group (P<0.001), in the TF-Amph group compared to TO-Amph group (P<0.001), and in the TF-Amph group than the TF-Sal group (P=0.001). Pre-exposure to amphetamine had no effects on basal ERK2 activation, as indicated by a lack of a difference in ERK2 activation between the TO-Sal and the TO-Amph groups (P=0.341).

3.2 AMPHETAMINE PRE-EXPOSURE INCREASES NAC ERK2 ACTIVATION DURING PIT AND POTENTIATES THE PIT EFFECT

To determine whether psychostimulant pre-exposure has an effect on CS-evoked NAc ERK2 activation after PIT, rats were treated with either amphetamine (1mg/kg i.p.) or saline (1ml/kg i.p.) immediately after every daily Pavlovian training session as described above. After 8 days of Pavlovian conditioning, all rats received instrumental training, starting with one day of FR1 followed by increasing random ratio schedules through RR3/RR8. Figure 5A shows the data for Pavlovian training, and the results are similar to those obtained in Experiment 1: on the first day of training, none of the four groups (TF-Amph, n=7; TF-Sal, n=6; TO-Amph, n=6 and TO-Sal, n=6) showed discriminative approach to the food cup. Both TF groups acquired discriminative food cup approach, and this effect was not influenced by drug treatment. ANOVA showed a significant group x day interaction ($F_{7,147}$ = 8.219; P<0.001), but no significant group x day x drug interaction ($F_{7,147}$ = 0.303; P=0.952). Post-hoc comparisons showed no difference in discriminative food cup approach on the first day of training, but on the 8th and last day of training, means for TF-Amph and TF-Sal groups were significantly higher than they were on day 1; (P<0.001) whereas the scores on day 8 for TO-Amph and TO-Sal controls did not differ from day 1 (P=1.0). Figure 5B shows that all groups learned to lever press during instrumental conditioning, as confirmed by a significant main effect of day ($F_{4,84}$ = 123.421; P<0.001), the rate of conditioning did not vary as a function of prior Pavlovian conditioning or drug treatment; the group x day interaction ($F_{4,84}$ = 0. 318; P=0. 865) and the day x group x drug interaction ($F_{4,84}$ =0.432; P=0.785) were not significant.

The day after completion of instrumental conditioning, rats were tested for PIT in extinction (lever presses did not result in the delivery of sucrose pellets). PIT is demonstrated by a higher rate of lever pressing during the CS compared with the rate of lever pressing during the preCS. Lever press rates during successive 90-sec blocks by a representative TF-Sal and a representative TF-Amph rat are shown in Figure 6A. Figure 6B shows the preCS and CS lever press rates for each group. TF groups but not TO groups displayed higher levels of lever pressing during the CS compared to the period preceding the CS (i.e., positive PIT). ANOVA confirmed a significant CS period x group interaction ($F_{1,21}$ = 43.924; P<0.001). Importantly, the difference between groups was dependent on drug treatment during Pavlovian conditioning, as demonstrated by a significant CS period x group x drug interaction ($F_{1,21}$ = 6.605; P=0.018). Post-hoc comparisons revealed that the PIT effect was present only in TF-Amph (P<0.001) and TF-Sal (P=0.022) groups but not in TO-Amph (P=0.927) or TO-Sal (P=0.148) groups, and that the lever-press rates during the CS period was significantly higher for the TF-Amph group than the TF-Sal group (P=0.003). In contrast, none of the groups differed with respect to the lever-press rate during the preCS (all P>0.3). Figure 6C shows that the groups also did not differ during the extinction phase at the beginning of the PIT test. ANOVA revealed a main effect of the 90-sec block ($F_{2,40}$ =15.767; P<0.001), but no block x group or block x group x drug interaction (all P>0.09), which suggests that all groups extinguished at the same rate. Figure 6 D shows that the level of discriminative food cup approach during the PIT test was significantly higher in TF groups than TO groups (main group effect, $F_{1,20}$ = 31.904; P<0.001), but it was not sensitive to prior drug

treatment, as indicated by a lack of significant group x drug interaction ($F_{1,20}$ = 0.669; P=0.423).

Immediately after the PIT test, brains were collected to asses ERK2 activation. Figure 7 shows that ERK2 activation in TF groups was higher than TO groups, as indicated by a significant effect of group ($F_{1,21}$ = 73.140, P<0.001). Importantly, the increase in NAc ERK2 activation depended on drug treatment during Pavlovian conditioning, as indicated by a significant interaction group x drug ($F_{1,21}$ = 6.363, P=0.020). Post-hoc comparisons confirmed that ERK2 was significantly higher in the TF-Amph group compared to TF-Sal (P=0.002). No difference in NAc ERK2 activation was detected between samples obtained from the TO-Sal and TO-Amph groups (P=1.000), which indicates that basal ERK2 activation was unaffected by prior drug treatment.

3.3 DELAYED AMPHETAMINE PRE-EXPOSURE FAILS TO INCREASE CUE-EVOKED ERK2 ACTIVATION

To determine whether the timing of psychostimulant pre-exposure is an important factor in the ability of the prior drug treatment to affect the magnitude of CS-evoked NAc ERK2 activation, rats were treated with either amphetamine (1mg/kg i.p.) or saline (1ml/kg i.p.) at a delay, namely 6 hr after daily Pavlovian conditioning session. As shown in Figure 8, on the first day of conditioning, none of the four groups (TF-Amph, n=6; TF-Sal, n=6; TO-Amph, n=6 and TO-Sal, n=6) showed discriminative approach to the food cup (more food cup approaches during the CS period compared to the preCS). TF groups developed discriminative food cup approach during the eight days of training,

but this effect did not differ between drug treatments, demonstrated by a significant group x day interaction ($F_{7,140}$ = 11.525; P<0.001) but no significant group x day x drug interaction ($F_{7,140}$ = 0.382; P=0.912). Post-hoc comparisons showed no difference in discriminative scores between groups during the first day of training. On the 8th and last day of training, means for TF-Amph and TF-Sal groups were significantly higher than those of the TO groups (P<0.001), whereas the means of the TO-Amph and TO-Sal controls did not differ from the values on day1. Two days after the last training and drug treatment, rats were tested in extinction. As expected, the level of discriminative food cup approach was significantly higher among TF groups than the TO (main group effect, $F_{1,20}$ = 57.493; P<0.001), but it was not sensitive to prior drug treatment, as indicated by a lack of significant group x drug interaction ($F_{1,20}$ = 0.129; P=0.723). Different from the differential trend we observed in Experiment 1, trial-by-trial analysis of discriminative food cup approach during the four trial test revealed no difference between the TF-Sal and the TF-Amph group on either the first (Student's t-test for independent groups, t_{10} = -0.2, P=0.845, two-tailed) or the last trial (t_{10}= -1.26, P=0.234, two-tailed) (Figure 8C)..

Immediately after the test, brains were collected to assess ERK2 activation. Figure 9 shows that ERK2 activation in the NAc tissue of the TF groups was higher than in the TO groups. Importantly, in contrast to the results from Experiment 1 (immediate amphetamine), ERK2 activation did not differ between the TF-Amph and TF-Sal groups. ANOVA showed a main effect of group ($F_{1,20}$ = 34.459, P<0.001) but no group x drug interaction ($F_{1,20}$ = 0.066, P=0.8). Post-hoc comparisons confirmed that ERK2 activation was significantly higher in the TF-groups than the TO-groups (P<0.001). Pre-exposure

to amphetamine had no effect on basal ERK2 activation, as indicated by a lack of a difference between the TO-Sal and TO-Amph groups (P=0.794).

3.4 DELAYED AMPHETAMINE PRE-EXPOSURE FAILS TO INCREASE NAC ERK2 ACTIVATION DURING PIT AND DO NOT POTENTIATES PIT

To determine whether the timing of psychostimulant pre-exposure is an important factor in the ability of the prior drug treatment to affect the magnitude of CS-evoked NAc ERK2 activation and subsequent PIT effect, rats were treated with either amphetamine (1mg/kg i.p.) or saline (1ml/kg i.p.) 6 hr after the daily Pavlovian conditioning session, as described above, and thereafter received instrumental training, as described for Experiment 2. Figure 10A, shows the data for Pavlovian training. On the first day of training, none of the four groups (TF-Amph, n=6; TF-Sal, n=6; TO-Amph, n=6 and TO-Sal, n=6) showed discriminative approach to the food cup. Both TF groups acquired discriminative food cup approach and this effect was not influenced by prior drug treatment. ANOVA showed a significant group x day interaction ($F_{7,140}$ = 7.859; P<0.001) but no significant group x day x drug interaction ($F_{7,140}$ = 1.242; P=0.284). Post-hoc comparisons showed no difference in discriminative food cup approach on the first day of training but on the 8th and last day of training, means for TF-Amph and TF-Sal groups were significantly higher than they were on day 1 (P<0.001) whereas the scores on day 8 for TO-Amph and TO-Sal controls did not differ from those on day 1 (P=1.0). Figure 10B shows that all groups learned to lever-press during instrumental conditioning as confirmed by a significant main effect of day ($F_{4,80}$ = 13.409; P<0.001),

but instrumental conditioning did not vary as a function of prior Pavlovian conditioning or drug treatment; the group x day interaction ($F_{4,80}$ = 0.938; P=0.446) and the day x group x drug interaction ($F_{4,80}$ =1.047; P=0.388) were not significant.

The day after completion of instrumental conditioning, rats were tested for PIT in extinction. Lever press rates during successive 90-sec blocks by a representative TF-Sal and a representative TF-Amph rat are shown in Figure 11A. Figure 11B shows the preCS and CS lever press rates. As was the case in Experiment 2, TF groups, but not TO groups, displayed higher levels of lever pressing during the CS compared to the preCS period. ANOVA confirmed a significant CS period x group interaction ($F_{1,20}$ = 62.747; P<0.001) but, different from Experiment 2, no CS period x group x drug interaction ($F_{1,20}$ = 1.386; P=0.253). Post-hoc analyses showed that the PIT effect was present only in the TF-groups (P<0.001) but not the TO-groups (P=1.0). Similar to what was observed in Experiment 2, no significant differences were found when comparing preCS lever-press rates between the groups. During the extinction phase at the beginning of the PIT test, ANOVA showed a main effect of each 90 sec block ($F_{2,40}$ =10.973; P<0.001) and a significant block x group interaction ($F_{2,40}$ =3.748; P=0.032) as shown in Figure 11C. Post-hoc comparisons revealed that the TF-groups exhibited higher lever-press rates than the TO-groups during the second 90-sec block of the extinction phase (P=0.031), but no group difference was present during the first or the third 90-sec blocks (P=0.843, and P=0.891, respectively). The block x group interaction was not drug-dependent, as confirmed by the lack of a block x group x drug interaction ($F_{2,40}$ =0.173; P=0.841).).Figure 11D shows that the level of discriminative food cup approach during PIT was significantly higher in TF groups than TO groups (main group

effect, $F_{1,20}$ = 108.800; P<0.001), but it was not sensitive to prior drug treatment, as indicated by a lack of a significant group x drug interaction ($F_{1,20}$ = 0.742; P=0.399)

Immediately after the PIT test, brains were collected to assess ERK2 activation. Figure 12 shows that ERK2 activation in TF groups was higher than in TO groups, as indicated by a significant effect of group ($F_{1,20}$ = 34.145, P<0.001). Importantly, in contrast to results from Experiment 1 (immediate amphetamine), ERK2 activation was comparable in the TF-Amph and the TF-Sal groups. ANOVA showed a main effect of group ($F_{1,20}$ = 34.459, P<0.001) but no group x drug interaction ($F_{1,20}$ = 0.040, P=0.843). ERK2 activation was significantly higher in TF- groups than TO-groups (P=0.001). The lack of a significant interaction indicates that exposure to amphetamine 6 hr after daily Pavlovian training had no effect on either basal or cue-evoked ERK2 activation.

4.0 DISCUSSION

Psychostimulants have been shown to potentiate PIT (Wyvell & Berridge, 2000; Wyvell & Berridge, 2001; Saddoris et al., 2011; Shiflett, 2012; Peciña et al., 2013; LeBlanc et al., 2013). Here, we showed that exposure to a low dose of amphetamine immediately after Pavlovian conditioning increased CS-evoked ERK activation in the NAc. This drug exposure regime also produced enhancement of CS-evoked ERK activation during PIT and, importantly, enhanced potentiation of PIT. Exposure to amphetamine 6 hours after Pavlovian conditioning, however, failed to increase CS-evoked ERK activation and also did not potentiate PIT.

Wyvell & Berridge (2000) studied the effect of amphetamine on PIT by exposing rats to a relatively high dose of amphetamine (3 mg/kg i.p.) for 6 days after Pavlovian and instrumental conditioning had ended (training and drug exposure did not overlap). They tested for PIT 10 days after the last drug treatment and observed a potentiation of the PIT effect in rats that had been treated with amphetamine. Using overall the same study design but exposing the rats to cocaine (15 mg/kg i.p.) instead of amphetamine, Leblanc et al. (2013) found a similar effect of psychostimulant exposure on subsequent PIT. Different from many of the previous studies, we tested whether amphetamine exposure affects PIT when administered immediately after daily Pavlovian training. We also examined whether the effect is different when amphetamine is administered at a

distinct delay after daily Pavlovian training. We found that a low dose of amphetamine (1 mg/kg i.p.) can potentiate PIT, but only if given immediately after Pavlovian training. We did not find evidence for enhanced PIT when the low dose of amphetamine was administered 6 hours after Pavlovian training.

Repeated administration of a psychostimulant can cause a progressively increased behavioral response to subsequent administrations of the same drug. This effect of addictive drugs is termed sensitization (Nishikawa et al., 1983; Robinson & Berridge, 1993). Wyvell & Berridge (2000) explained the potentiating effect of psychostimulant exposure on PIT in terms of the Incentive-Sensitization theory of addiction, which postulates that sensitization to drugs and drug-associated cues is due to increases in the incentive salience or motivational value of reward-associated stimuli by enhanced dopaminergic neurotransmission (Robinson & Berridge, 1993). Wyvell & Berridge (2001), Peciña et al. (2013), and LeBlanc et al. (2013) all found that rats exposed repeatedly to psychostimulants exhibited enhanced PIT. Pressing on the reward-associated lever was augmented only in presence of the CS, consistent with the idea that prior psychostimulant exposure increases the motivational incentive salience of the CS. Similar to results of Wyvell & Berridge (2001), Peciña et al. (2013), and LeBlanc et al. (2013), we did not observe increased pressing of the inactive lever or of the active lever in the absence of the CS by rats previously exposed to amphetamine. Thus, the drug regime we employed did not seem to cause an increase in general arousal. However, the fact that we observed potentiation of PIT when rats received amphetamine immediately after the Pavlovian training session, but not when they received amphetamine 6 hours after the training session, suggests that the underlying

mechanism is different from the mechanism(s) that resulted in PIT potentiation when the psychostimulant exposure was separated temporally from both the Pavlovian and the instrumental conditioning phases (Wyvell & Berridge, 2000; Wyvell & Berridge, 2001; Shiflett et al., 2012; Peciña et al., 2013; LeBlanc et al., 2013). Thus, whereas potentiation of PIT in those instances may stem from drug-induced sensitization of incentive salience, a different and possibly more specific mechanism appears to underlie the drug-induced enhancement of PIT observed in our studies. One possible candidate is an effect of amphetamine on memory consolidation.

Psychostimulants have been shown to work as “memory boosters” in that their administration was found to enhance different types of memory. A memory enhancing effect has been shown mostly in associative memories, such as fear and appetitive Pavlovian conditioning paradigms (Wood & Anagnostaras, 2009; Simon & Setlow, 2006). That psychostimulants can strengthen memory formation is not surprising: psychostimulants cause a rapid increase in ERK activation in many regions of the brain, including in limbic regions involved in memory formation and in elements of the reward system involved in reward learning (Mao et al., 2012; Valjent et al., 2000; Valjent et al., 2004). Memory formation is also accompanied by an increase in ERK activation in the areas critical for the respective types of memory. Spatial learning, for example, depends on ERK activation in the hippocampus (Kelly et al., 2003). Interestingly, blockade of ERK activation was found to prevent memory formation only when ERK inhibition occurred shortly after exposure to the spatial information (i.e., the learning experience) but not when it occurred hours after the learning experience (Kelly et al., 2003). These findings suggest that ERK is required early during the consolidation process. The same

time-dependence of the disruptive effect of ERK blockade is observed in experiments investigating long-term potentiation (LTP), the hypothesized synaptic correlate of memory formation. Blockade of ERK shortly after LTP-inducing stimulation prevents the development of LTP. However, after LTP has been established, ERK inhibition has no effect on the potentiated response (Toyoda et al., 2007).

It is noteworthy that the decline of discriminative approach to the food cup during testing of the Pavlovian conditioned response after Pavlovian conditioning proceeded slightly differently in saline-treated and amphetamine-treated conditioned rats. Whereas rats in the TF-Sal group exhibited a much lower level of discriminative approach behavior on the last trial than on the first trial of the test (which was conducted in extinction), rats in the TF-Amph group exhibited a relatively high level of discriminative approach behavior throughout the test. Moreover, we did not find differential extinction of discriminative approach behavior when amphetamine was administered 6 hours after Pavlovian conditioning, a time when the early phase of consolidation likely had been completed. Together, these findings are consistent with the possibility that the CS-outcome (i.e., tone-food) association was slightly stronger in the TF-Amph group than the TF-Sal group.

In our experiments, we observed a clear difference in PIT potentiation as a function of the timing of amphetamine administration relative to the Pavlovian conditioning session (i.e., the associative learning experience). When amphetamine was administered immediately after the session, we found robust potentiation of PIT; whereas when the drug was administered 6 hours after the Pavlovian conditioning session, the magnitude of the PIT effect was similar to that observed in drug-naïve rats.

The dependence of PIT potentiation on the timing of amphetamine administration argues against increased general arousal or sensitization of incentive salience as the underlying cause of the amphetamine-induced enhancement of PIT in our situation. Our failure to observe an increase in pressing of the active lever in the absence of the CS in the amphetamine-treated groups, and our failure to observe an increase in pressing of the inactive lever in the amphetamine-treated groups also argue against an explanation in terms of enhanced general arousal or general sensitization to stimuli, such as the sight of a reward-paired lever. Our findings that the same dose of amphetamine that attenuates extinction of discriminative responding and produces strong potentiation of PIT when given immediately after Pavlovian training fails to have these effects when given 6 hours after Pavlovian training suggest that amphetamine may have acted on consolidation of the cue-reward association, and that enhanced consolidation of the association played a role in the enhancement of the PIT effect. Thus, given the role of ERK in memory consolidation, and the relation between psychostimulants and ERK, our data taken together suggest that one mechanism by which amphetamine may cause potentiation of PIT is through strengthening of the consolidation of the cue-reward association.

Our TO-Amph control rats, which received the same experience (tone exposure, instrumental conditioning and drug exposure) as our TF groups, except for the cue-reward association, did not show an increase in ERK activation in the NAc when compared to TO-Sal controls. In contrast, amphetamine treatment had a clear effect on NAc ERK activation in the TF groups. As we showed previously (Shiflett et al., 2008; Remus & Thiels, 2013), ERK activation is increased during PIT, and this increase in

ERK activation is specifically driven by the CS. Previous work (Nishikawa et al., 1983) showed that chronic psychostimulant exposure induces an increase in evoked dopaminergic transmission. More recently, D1R activation has been linked to increased ERK activation in striatum (Haberny & Carr, 2005, Cerovic et al., 2013, Shi & McGinty, 2011). Moreover, Wan & Peoples (2006) showed that excitatory responses to a CS within the NAc were increased after chronic amphetamine treatment. Therefore, it seems reasonable to conclude that the potentiation of PIT after repeated amphetamine exposure reflects a chronic drug effect on CS-evoked dopamine release, NAc cell firing, and ERK signaling (Wassum et al., 2013; Argona et al., 2009; Cheng et al., 2003; Saddoris et al., 2001; Remus & Thiels, 2013; Shiflett et al., 2008).

We showed increased CS-evoked ERK activation in the NAc during PIT in rats that previously had received repeated injections of amphetamine. In sensitized rats exposed to a challenge injection of amphetamine, ERK signaling was found to lie downstream of D1R activation (Haberny & Carr, 2005; Shi & McGinty, 2011; Durieux et al., 2012). Remus et al. (SfN, 2012) demonstrated that CS-evoked ERK activation is increased specifically in medium spiny neurons (MSNs) that express D1R markers. It would be interesting to study whether the CS-evoked increase in ERK activation observed after amphetamine exposure during Pavlovian conditioning also is regulated through D1R signaling and occurs preferentially or exclusively in D1 receptor-containing MSNs. It also would be interesting to determine whether the enhancement of CS-evoked ERK activation during PIT after repeated amphetamine shows subregional specificity (i.e., is more pronounced in the shell or the core of the NAc). Past studies have not found a differential presence of activity-related markers (i.e., fos) within a

specific subregion of the NAc after PIT, whether in drug-naïve rats (Remus & Thiels, 2013) or in rats that had repeatedly been exposed to amphetamine prior to training and testing (Peciña et al., 2013). Information on specific regional ERK activation and neuronal phenotype would help identify the neurobiological targets that are affected during chronic psychostimulant exposure.

Our data do not demonstrate a definitive role of ERK in the potentiation of PIT by amphetamine exposure, but they are strongly suggestive. Daily administration of a low dose of amphetamine did not have an effect on basal ERK phosphorylation, but the enhancement of ERK activation depended on exposure to a CS, i.e., a cue previously paired with reward. Furthermore, an effect of amphetamine on PIT was observed only when it also caused an increase in CS-evoked ERK activation, but no effect on PIT was observed when the psychostimulant was delivered at a delay and did not cause an enhancement of PIT.

In summary, our work suggests ERK as a possible mediator of enhanced PIT after chronic drug exposure. The effect of psychostimulants on consolidation of cue-reward association may contribute to exaggeration of cue-control over behavior after chronic drug exposure. Understanding the molecular mechanisms that underlie the potentiating effect of psychostimulants on reward-seeking will be valuable in the design of more effective treatments for relapse in drug abuse.

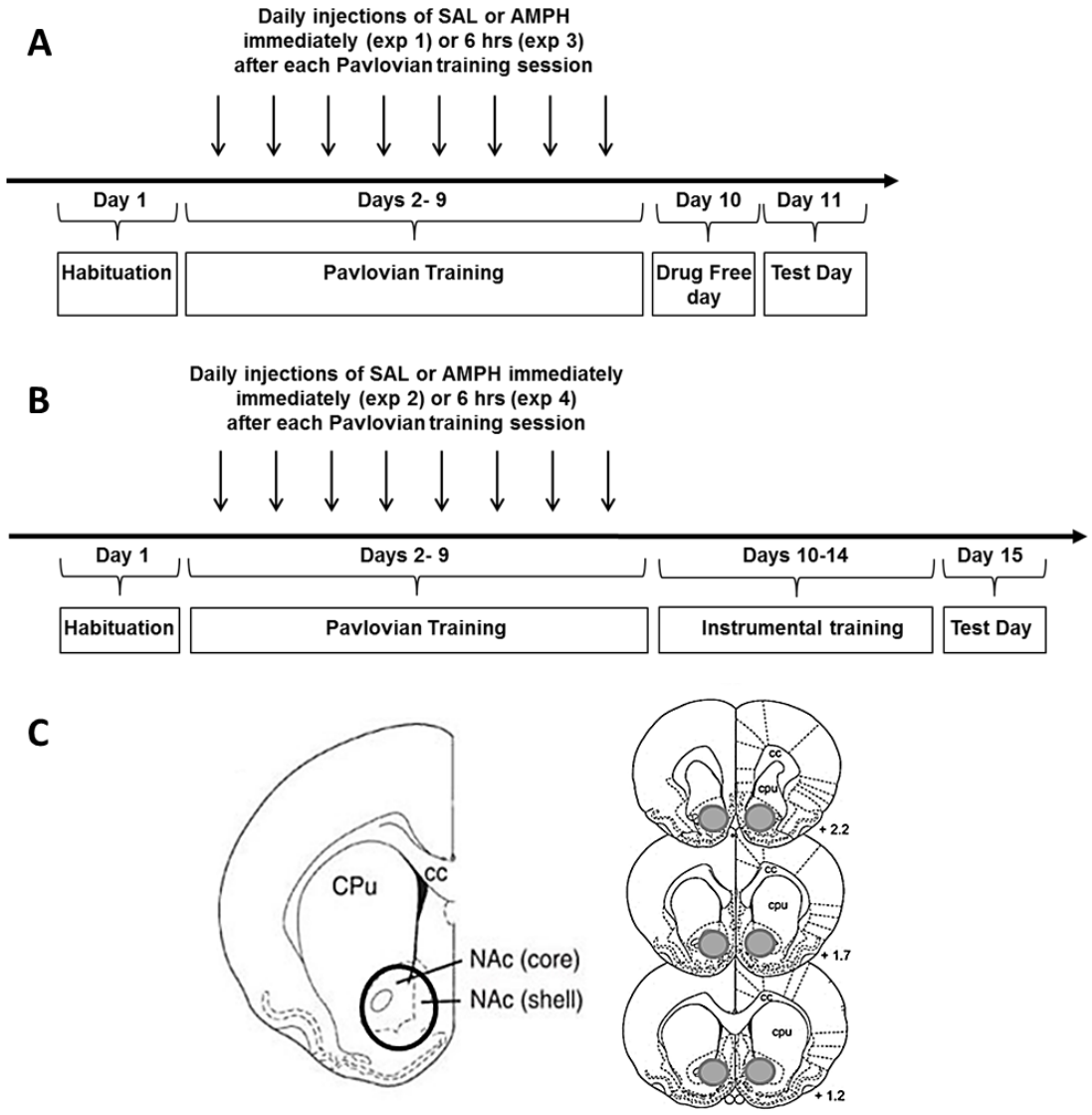

Figure 1 Timeline for behavioral experiments.

A-Timeline for Pavlovian conditioning experiments for 0 hrs (immediate) or 6 hrs (delayed) amphetamine treatment. Rats were injected with AMPH or SAL immediately after the end of each conditioning session. Testing was conducted in extinction, forty-eight hrs after the last drug treatment. B-Timeline for Pavlovian Instrumental Transfer (PIT) experiments; the day after the last Pavlovian training and drug exposure, rats were trained to lever press for food and transfer was measured on day 15 in extinction. C- NAc punch positioning (left panel) and areas sampled for western blot analysis with a 2mm punch (right panel, shown in gray). Numbers indicate Paxinos's coordinates relative to bregma.

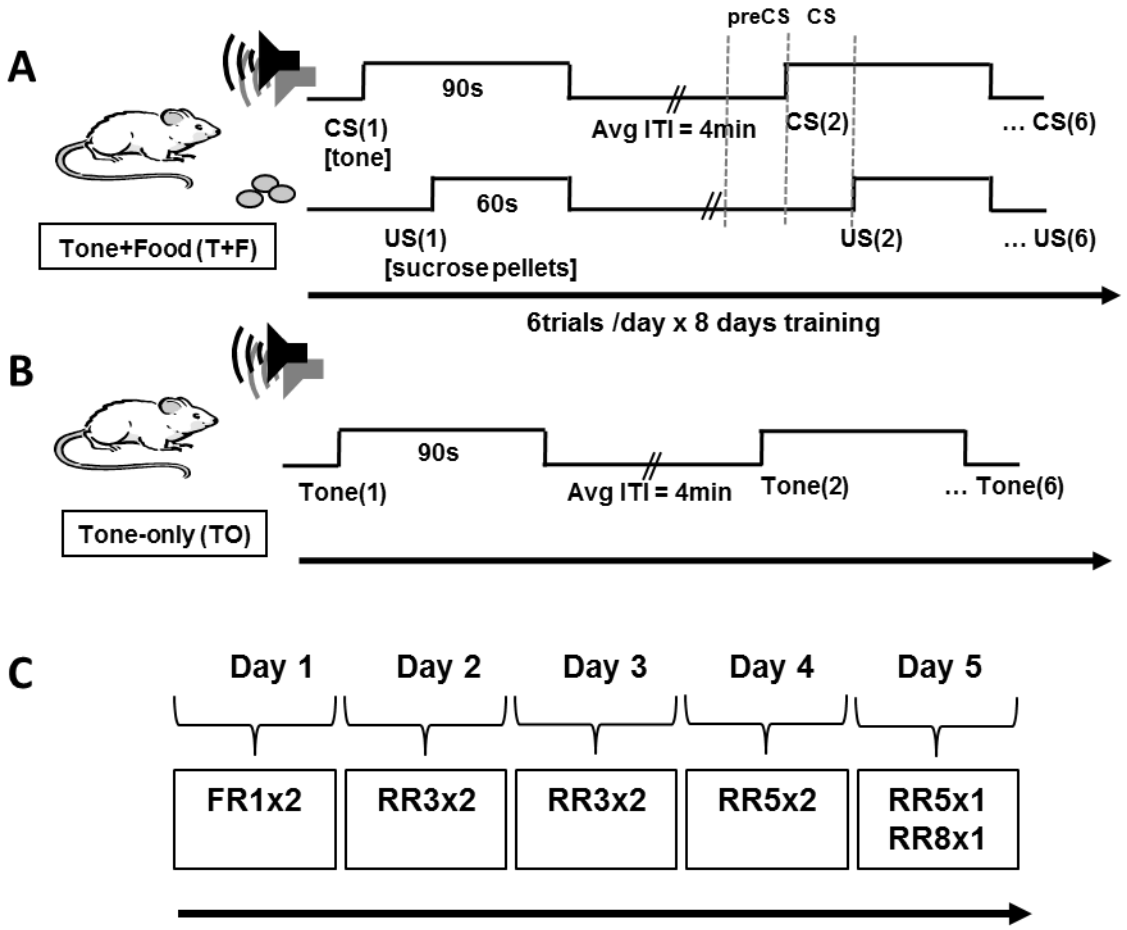

Figure 2 Behavioral design

Pavlovian trials design. Food delivery was preceded by a 3kHz tone. Each session had 6 Tone-Food presentation trials with a variable ITI averaging 4 min (3.5-5.5 min). Appetitive Conditioning was measured by food cup approach counts during the first 30 seconds of CS presentation minus food cup approach during the 30 seconds of preCS period (baseline). Shown in A are the design for Tone+Food groups and in B design for Tone-only groups. C- Instrumental training sequence progression from FR1 to RR8.

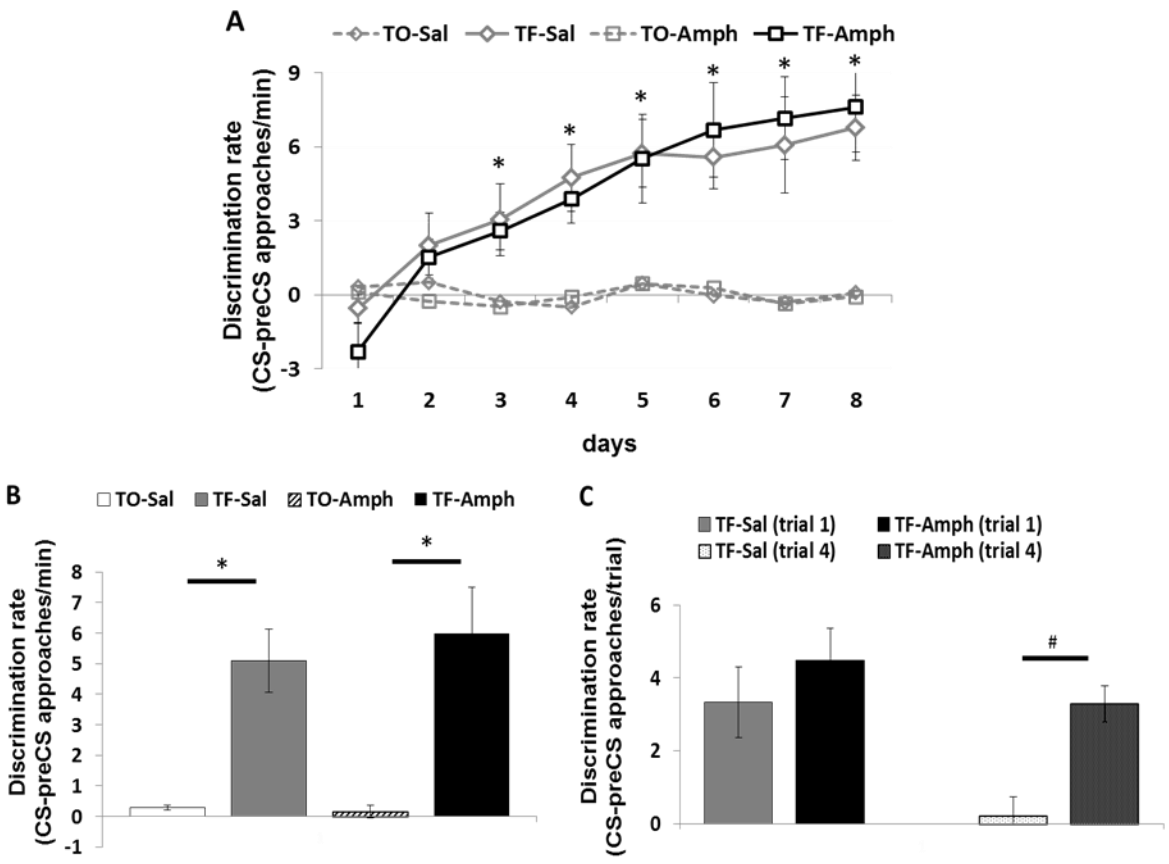

Figure 3 Conditioned food cup approach during Pavlovian training and testing is not affected by immediate post-training exposure to amphetamine

A- Means ± s.e.m. of discriminative food cup approach for the TO-Sal (n=7), TO-Amph (n=7), TF-Sal (n=7) and TF-Amph (n=7) groups across days of Pavlovian training. B- Means ± s.e.m. of discriminative food cup approach during test day. Drug was delivered after the training and testing was performed in drug-free conditions. Food was available during training but not during testing. C- Means ± s.e.m of food cup approaches during trials 1 and 4 of the test. TF-Amph and TF-Sal groups did not differ during trial one. TF-Sal group approached the food cup fewer times than TF-Amph group during trial four.

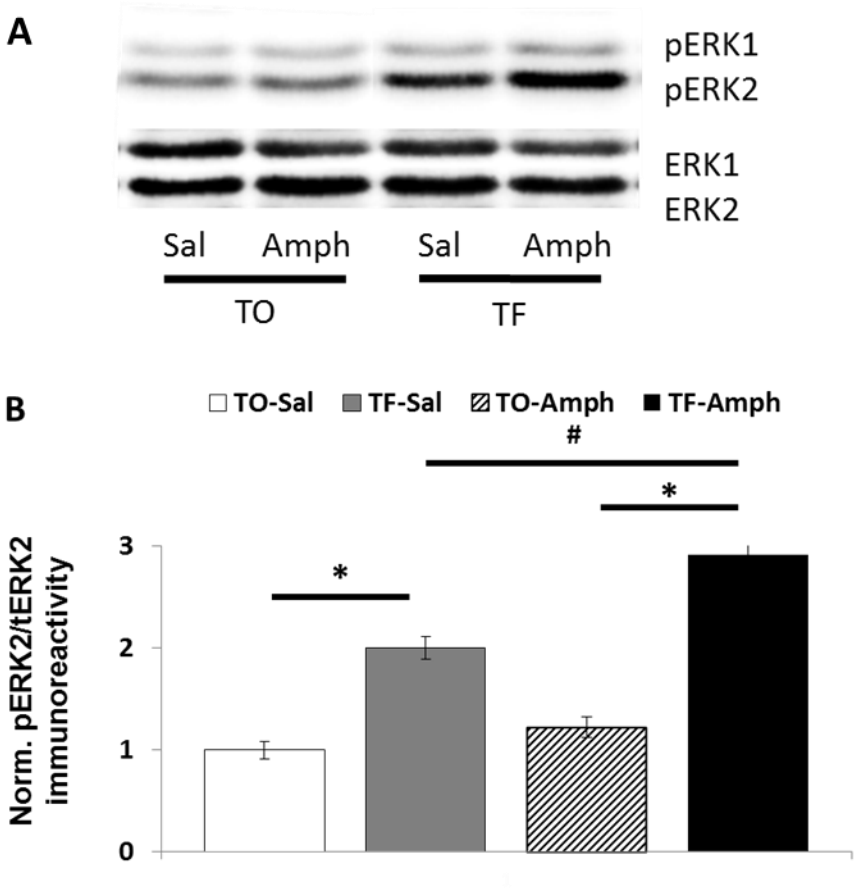

Figure 4 Post-training exposure to amphetamine increases cue-evoked ERK activation

. A- Representative blots for the TO-Sal (n=7), TO-Amph (n=7), TF-Sal (n=7) and TF-Amph (n=7) groups. B- Means ± s.e.m. of densitometric analysis of pERK2 immunoreactivity normalized to tERK2 immunoreactivity in NAc tissue from rats whose behavioral results are shown in Fig 3. Rats that were exposed to TF pairings exhibit higher pERK/tERK ratios (* indicates $p<0.05$ for TF vs TO groups). Exposure to amphetamine potentiates this activation (# indicates $p<0.05$ for TF-Amph vs TF-Sal comparison).

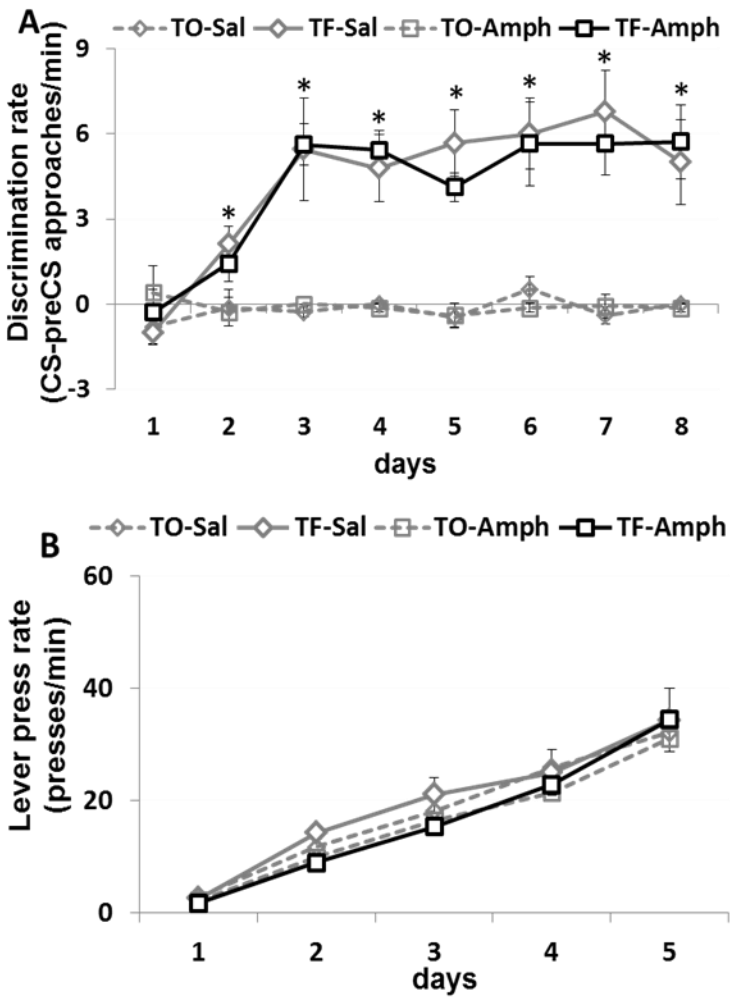

Figure 5 Immediate post-training exposure to amphetamine does not affect conditioned response performance (A) or acquisition of a instrumental behavior (B)

A- Means ± s.e.m. of discriminative food cup approach for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=7) groups across days of Pavlovian training. B- Means ± s.e.m. of lever pressing during instrumental conditioning. Drug was delivered only after the Pavlovian training. Instrumental conditioning was performed in drug-free conditions. Food was available during training.

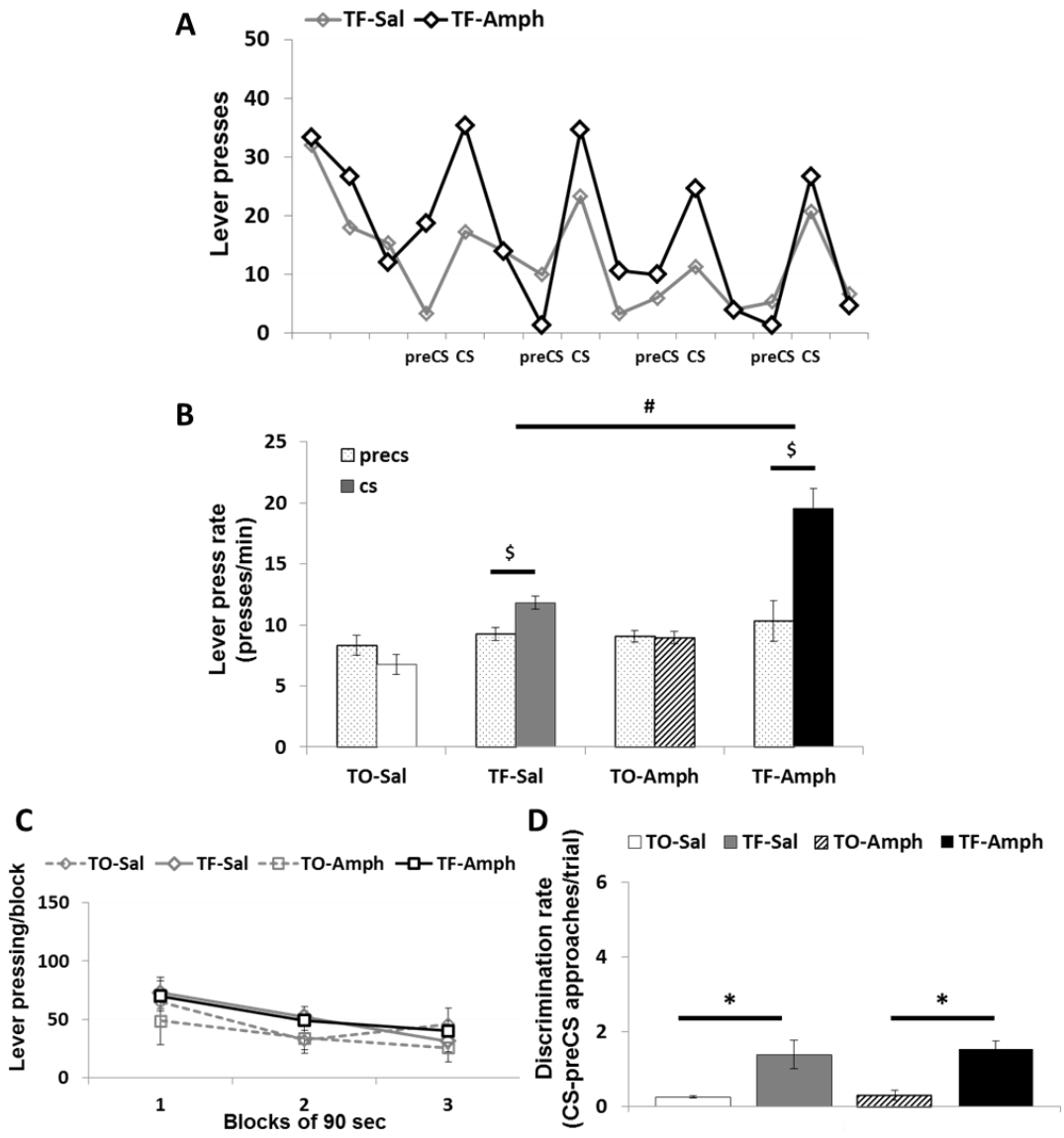

Figure 6 Immediate post-training exposure to amphetamine potentiates PIT

A- Example of a TF-Sal and a TF-Amph subject during PIT testing: presence of the food cue enhances lever pressing and this enhancement is potentiated in rats that were treated with AMPH during Pavlovian conditioning. B-Shown are means ± s.e.d. of lever pressing rates during preCS and CS periods. Testing was performed in drug-free conditions and food was not available. Only TF paired rats increased lever pressing when the cue was present (PIT; $ indicates $p<0.05$ for CS vs preCS rates). This effect was significantly higher on rats pretreated with amphetamine (# indicates $p<0.05$ for TF-Sal vs TF-Amph groups comparison). C- Lever pressing rates (in extinction) during the early phase of the PIT test, no differences between groups were found. D- Food cup approach rates during PIT testing. Only TF groups displayed conditioned approach and pre-exposure to AMPH had no effect.

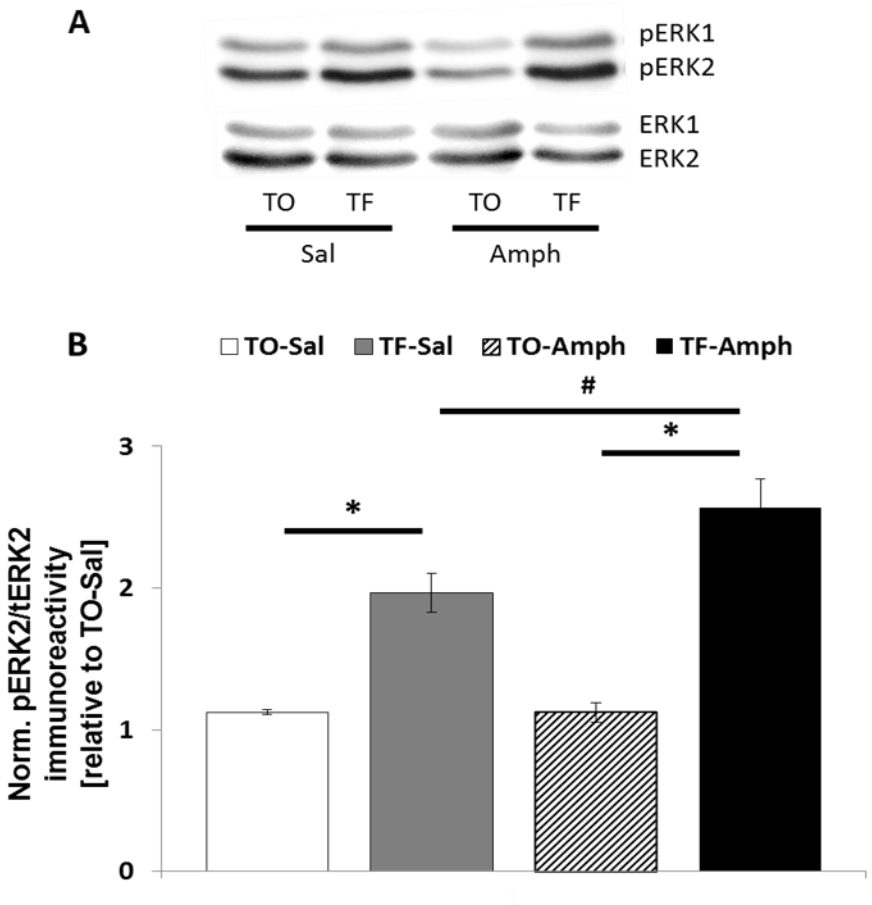

Figure 7 Post-training exposure to amphetamine increases cue-evoked ERK activation during PIT

A- Representative blots for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=7) groups. B- Means ± s.e.m. of densitometric analysis of pERK2 immunoreactivity normalized to tERK2 immunoreactivity in NAc tissue from rats whose behavioral results are shown in Fig 6. Rats that were exposed to TF pairings exhibit higher pERK/tERK ratios (* indicates $p<0.05$ for TF vs TO groups). Exposure to amphetamine potentiates this activation (# indicates $p<0.05$ for TF-Amph vs TF-Sal comparison).

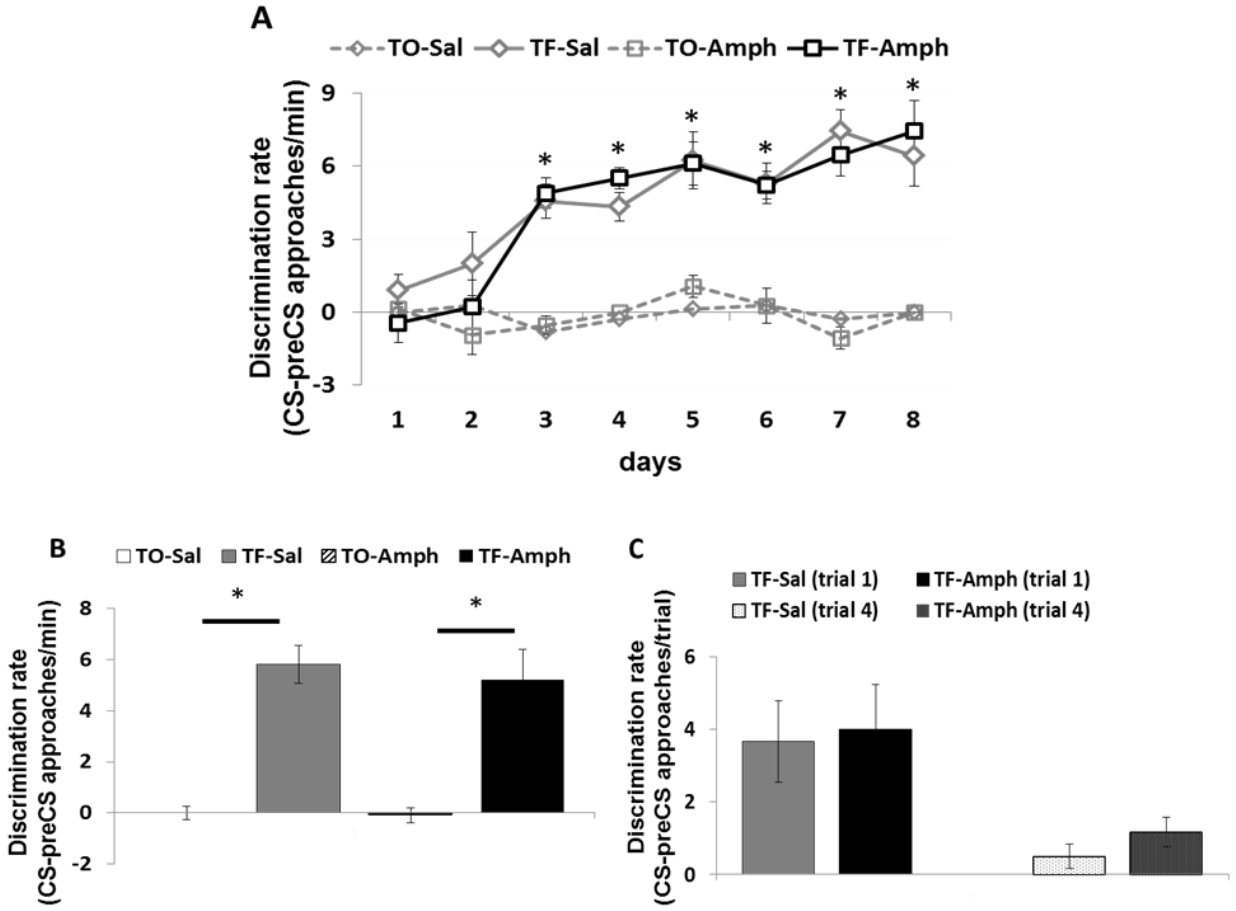

Figure 8 Conditioned food cup approach during Pavlovian training and testing is not affected by 6 hrs post-training exposure to amphetamine

A- Means ± s.e.m. of discriminative food cup approach for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=6) groups across days of Pavlovian training. B- Means ± s.e.m. of discriminative food cup approach during test day. Drug was delivered after the training and testing was performed in drug-free conditions. Food was available during training but not during testing. C- Means ± s.e.m of food cup approaches during trials 1 and 4 of the test. TF-Amph and TF-Sal groups did not differ during either trial.

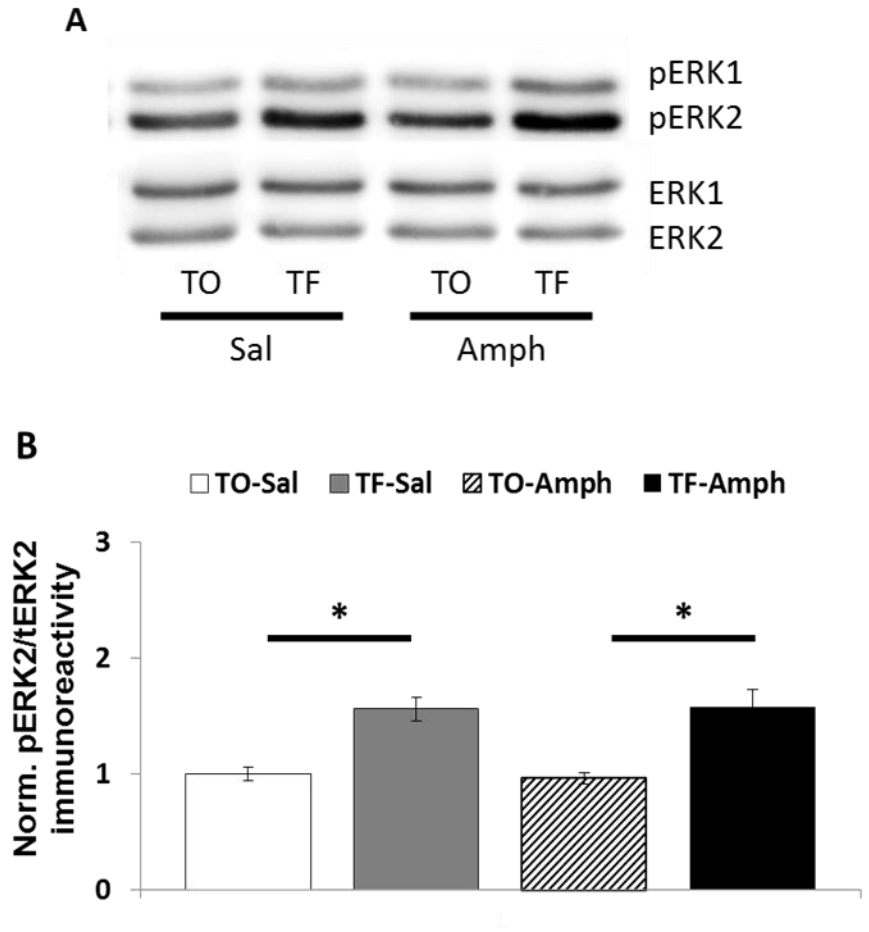

Figure 9 Delayed (6 hrs) post-training exposure to amphetamine does not increase cue-evoked ERK activation

A- Representative blots for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=6) groups. B- Means ± s.e.m. of densitometric analysis of pERK2 immunoreactivity normalized to tERK2 immunoreactivity in NAc tissue from rats whose behavioral results are shown in Fig 8. Rats that were exposed to TF pairings exhibit higher pERK/tERK ratios (* indicates $p<0.05$ for TF vs TO groups).

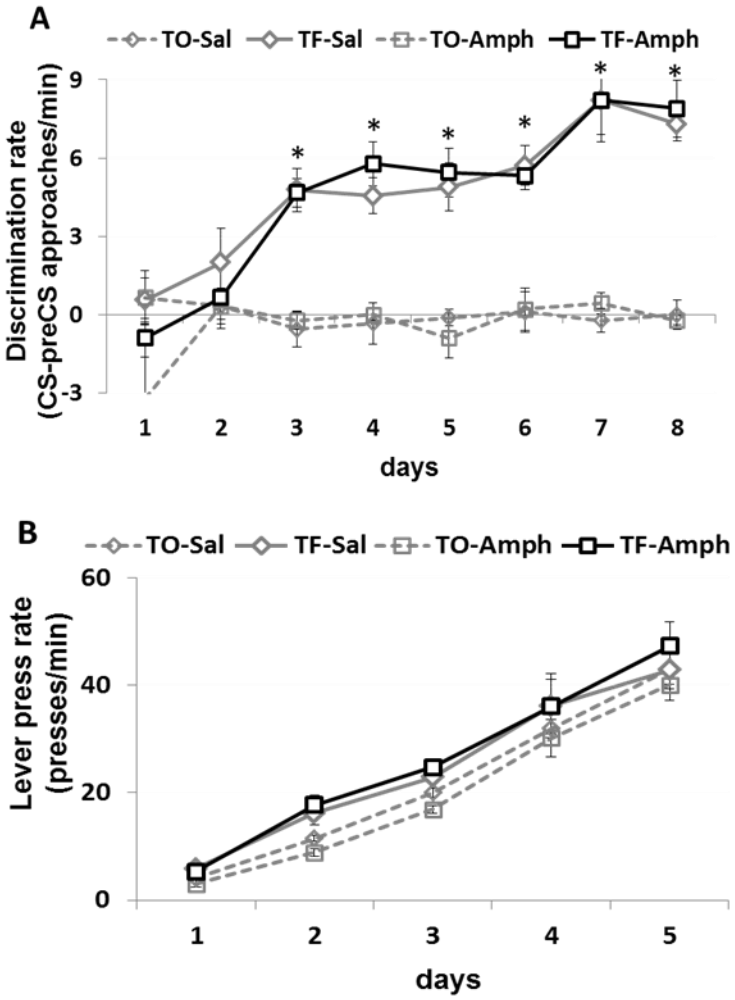

Figure 10 Delayed post-training exposure to amphetamine does not affect conditioned response performance (A) or acquisition of an instrumental behavior (B)

A- Means ± s.e.m. of discriminative food cup approach for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=6) groups across days of Pavlovian training. B- Means ± s.e.m. of lever pressing during instrumental conditioning. Drug was delivered only after the Pavlovian training. Instrumental conditioning was performed in drug-free conditions. Food was available during training.

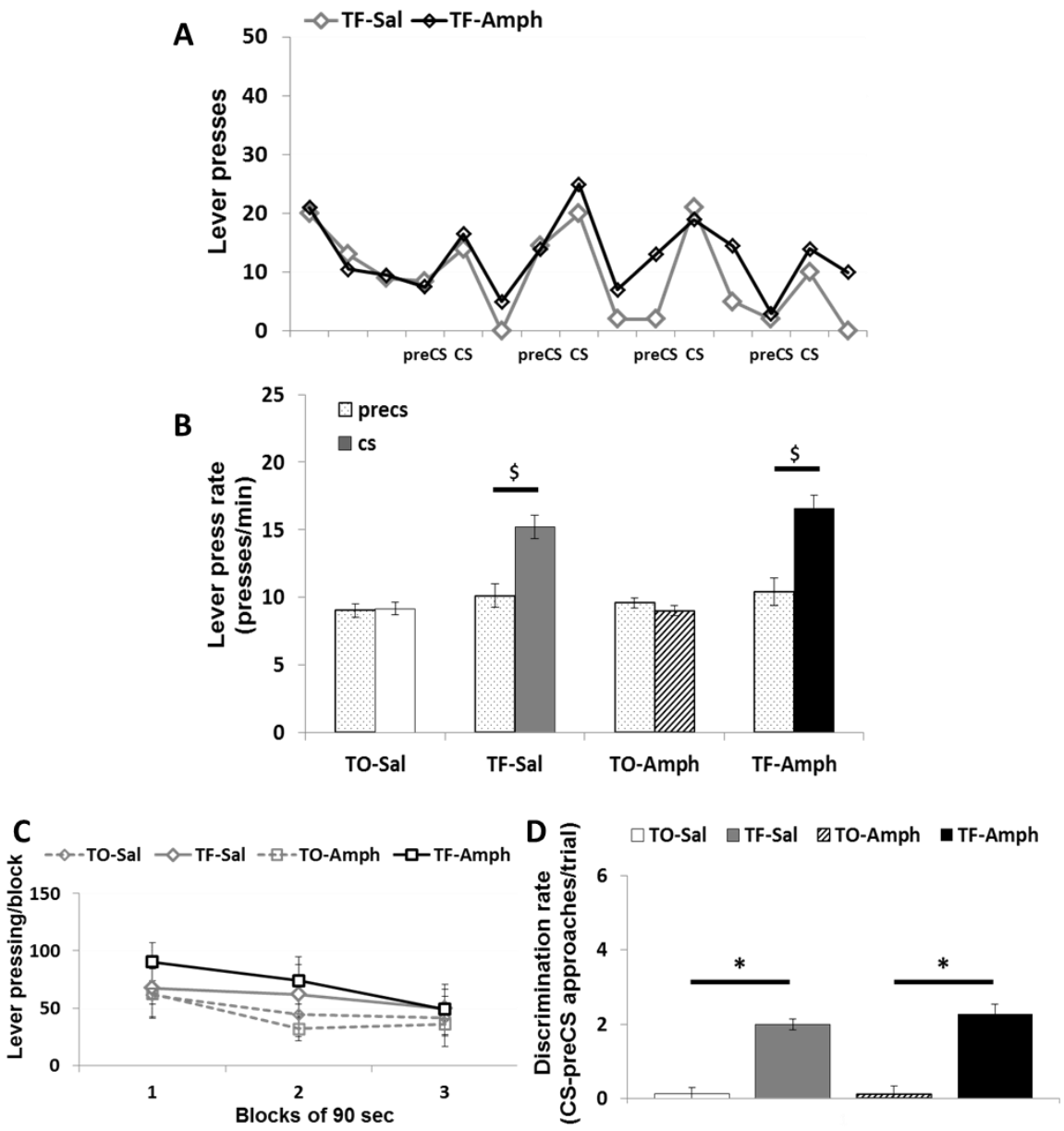

Figure 11 Delayed post-training exposure to amphetamine fails to potentiate PIT

A- Example of a TF-Sal and a TF-Amph subject during PIT testing: presence of the food cue enhances lever pressing. B-Shown are means ± s.e.d. of lever pressing rates during preCS and CS periods. Testing was performed in drug-free conditions and food was not available. Only TF paired rats increased lever pressing when the cue was present (PIT; $ indicates $p<0.05$ for CS vs preCS rates). This effect was not potentiated on rats pretreated with amphetamine. C- Lever pressing rates (in extinction) during the early phase of the PIT test, no differences between groups were found. D- Food cup approach rates during PIT testing. Only TF groups displayed conditioned approach and pre-exposure to AMPH had no effect.

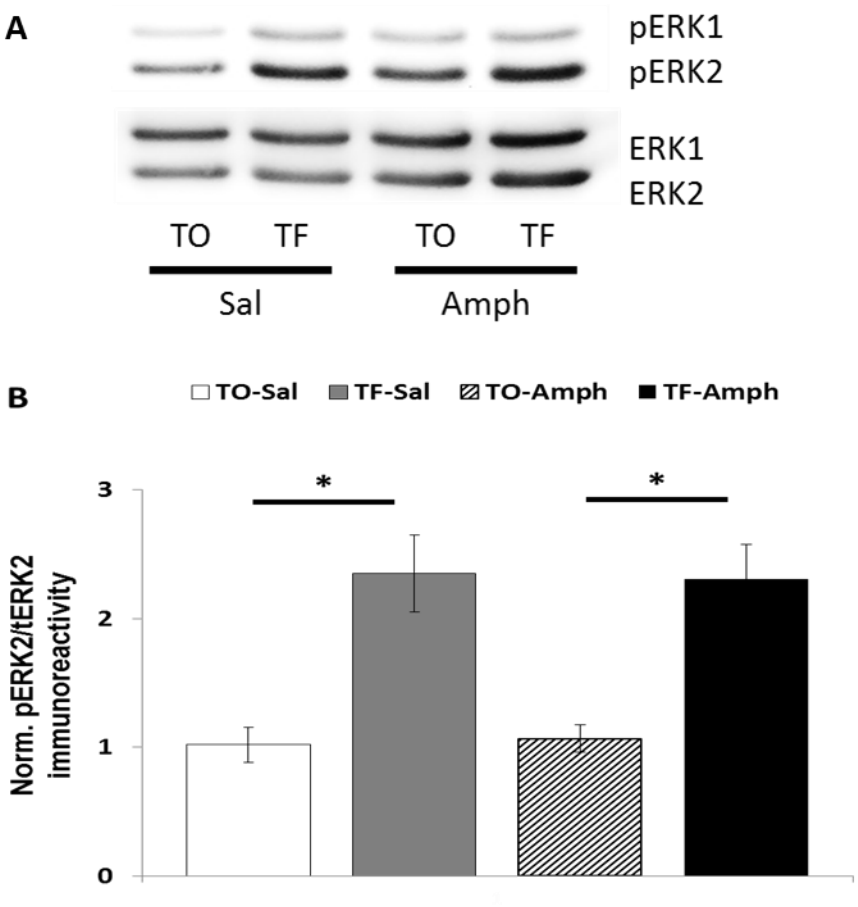

Figure 12 Delayed post-training exposure to amphetamine does not increase cue-evoked ERK activation during PIT

A- Representative blots for the TO-Sal (n=6), TO-Amph (n=6), TF-Sal (n=6) and TF-Amph (n=6) groups. B- Means ± s.e.m. of densitometric analysis of pERK2 immunoreactivity normalized to tERK2 immunoreactivity in NAc tissue from rats whose behavioral results are shown in Fig 6. Rats that were exposed to TF pairings exhibit higher pERK/tERK ratios (# indicates $p<0.05$ for TF vs TO groups).

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